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In Vitro Rearing of *Edovum puttleri*, an Egg Parasitoid of the Colorado Potato Beetle, on Artificial Diets: Effects of Insect Cell Line-Conditioned Medium

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Effects of conditioned media prepared from cell lines derived from 11 insect species (six families, three orders) on the in vitro growth and development of the egg parasitoid *Edovum puttleri* were investigated. The parasitoid exhibited significantly different responses to the various insect cell line-conditioned media that were incorporated into the artificial diets. When cell lines were derived from embryos, higher percentages of 3rd instars and prepupae were observed than when cell lines were derived from fat body or ovaries. Medium conditioned with cell line IPLB-CPB2 derived from the embryos of the Colorado potato beetle produced the best result. Preconditioning time was important. In general, 5 days of preconditioning appeared to be optimal. The growth- and development-promoting effect may have resulted from growth factors or growth-supporting factors produced/ released by the insect cell lines into the culture medium. Upon storage at 0–4°C for 7–14 days, the ability of cell line-conditioned medium to promote development beyond the second instar was greatly reduced (approximately 10–55%). Our studies demonstrated that to support the in vitro growth and development of *E. puttleri*, insect hemolymph could be successfully replaced with insect cell line-conditioned medium. These findings should facilitate the development of a cost-effective mass-rearing system for *E. puttleri* and/or other parasitoids. Arch. Insect Biochem. Physiol. 40:173–182, 1999. **Published 1999 Wiley-Liss, Inc.†**

Key words: artificial diet; insect cell line; in vitro culture; *Edovum puttleri*; parasitoid development

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Abbreviations used: CPB = Colorado potato beetle.

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INTRODUCTION

Edovum puttleri, an eulophid egg parasitoid, is an important potential biological agent for controlling the Colorado potato beetle (CPB), *Leptinotarsa decimlineata* (Coleoptera: Chrysomelidae), one of the most destructive insect pests of potato, tomato, and eggplant crops in North America and Europe (Schalk and Stoner, 1979; Hare, 1980; Puttler and Long, 1983). The parasitoid has been used to successfully control CPB in New Jersey eggplant fields with significantly reduced application of pesticides (Williams, 1987). Beetle eggs in the field were parasitized at a rate of 46.9%, while 73.9% of the beetle eggs in egg masses were destroyed by mass-released *E. puttleri* (Williams, 1987). The cost of mass-rearing this parasitoid on CPB eggs is high, and hence, an in vitro rearing system would facilitate the cost-effective use of *E. puttleri* to control CPB. An artificial diet has been developed in our laboratory, which successfully supports the development of this wasp from the egg through the pupal stage (Hu et al., 1998). However, the diet requires the addition of *Manduca sexta* larval hemolymph (Hu et al., 1998). Since this requirement for hemolymph increases the difficulty and cost of making artificial diet, it is imperative to find an alternative for insect hemolymph in the diet.

Ferkovich et al. (1991) reported that growth factors or growth-supporting materials released by a fat body cell line of gypsy moth (*Lymantria dispar*) improved the embryonic development of *Microplitis croceipes,* a larval endoparasitoid of *Heliothis* spp. Based on their findings, we decided to investigate the effects of medium conditioned with various insect cell lines in promoting the in vitro growth and development of *E. puttleri*. If insect cell line-conditioned medium could be substituted for insect hemolymph, it would facilitate the production of an artificial diet. The cost would be lowered because the techniques for large-scale production of insect cell lines have been developed (Lynn, 1996) and a serum-free medium is now available for maintaining cell lines (Komiya et al., 1998).

MATERIALS AND METHODS

Insects

Colorado potato beetles (CPBs) were reared on potato plants in a constant temperature growth chamber at 24 ± 1°C, r.h. of 70%, L:D regimen of 14:10 and light intensity of 600 lux. Each day, new plants were placed in rearing cages and CPB eggs were collected. The method of rearing *E. puttleri* was derived from that of Palmer (1996). The parasitoid was reared on CPB eggs and maintained in 1-gal (4 liters) glass jars at 24 ± 1°C, L:D regimen of 14:10, light intensity 1,750 lux. Adults were provided with pure honey and water. Since exposure to 7–14-day-old adult females results in the highest rates of parasitization (Lashomb et al., 1987), wasps of this age were used for colony maintenance and experiments.

Maintenance of Insect Cell Lines and Preparation of Preconditioned Media

Twelve cell lines derived from 11 species, representing six families and three orders, were used in these investigations (Table 1). Ten cell lines were from embryos, one was from fat body, and one was from ovaries. These cell lines were originally established as described by Lynn and Hung (1991), Lynn and Stoppleworth (1981), Lynn et al. (1988), Lynn and Shapiro (1997, 1998), Rochford et al. (1984), and Sieburth and Maruniak

TABLE 1. Culture Media for Various Insect Cell Lines

Medium	Cell line	Insect	Origin of cell lines
Ex-Cell 400	IPLB-DU182A	*Diabrotica undecimpunctata* (Coleoptera: Chrysomelidae)	Embryos
	IPLB-LdElta	*Lymantria dispar* (Lepidoptera: Lymantriidae)	Embryos
	IPLB-LdFB	*L. dispar*	Fat body
	IPLB-Tcon1	*Trichogramma confusum* (Hymenoptera: Trichogrammatidae)	Embryos
	IPLB-Tex2	*T. exiguum* (Hymenoptera: Trichogrammatidae)	Embryos
TNM-FH	IPLB-CPB2	*Leptinotarsa. decemlineata* (Coleoptera: Chrysomelidae)	Embryos
	IPLB-PiE	*Plodia interpunctella* (Lepidoptera: Pyralidae)	Embryos
	IAL-TN-R[2]	*Trichplusia ni* (Lepidoptera: Noctuidae)	Embryos
TC-100[a]	UFL-AG-286	*Anticarsia gemmatalis* (Lepidoptera: Noctuidae)	Ovaries
	IPLB-HvE6s	*Heliothis virescens* (Lepidoptera: Noctuidae)	Embryos
	IPLB-OIE505s	*Orgyia leucostigma* (Lepidoptera: Lymantriidae)	Embryos
	IPLB-PxE2	*Pluttella xylostella* (Lepidoptera: Pluteliidae)	Embryos

[a]Modified by Lynn (1996).

(1988). Cells were maintained in suspension and were subcultured weekly as described by Lynn (1996). The initial number of cells was approximately 1.5×10^4/ml. The basal medium for TNM-FH (supplemented Graces) and TC-100 (modified as in Lynn, 1996) were obtained from Gibco BRL (Gaithersburg, MD) and were supplemented with 9% (v/v) fetal bovine serum (Intergen, Purchase, NY). ExCell 400 was purchased from JRH Biosciences (Lenexa, KS). To ensure normal cell growth, medium was preconditioned with the desired cell line prior to use. In all cases except for one (CPB embryonic cells), approximately 15,000 cells (cell counts were determined using a hemocytometer) were present per ml of culture medium. Since CPB embryonic cells tended to form aggregates, it was impossible to acquire accurate cell counts for this cell line. The conditioned medium was used to prepare the artificial diet as described below. Conditioned medium did not contain cells except for those experiments in which the effects of diets with or without cells were being compared.

Preparation of Diets

Based on results from our previous studies (Hu et al.,1998), we prepared diets that consisted of 30% cell-line-conditioned medium and 70% fresh chicken egg yolk. Prior to collecting egg yolk, a fresh chicken egg was surface sterilized in 70% ethanol for 10 min. Using a 60-ml sterile syringe, the egg white was removed and then egg yolk was collected with a 1-ml sterile syringe. In order to avoid coagulation, yolk was immediately added to fresh culture medium or cell line-conditioned medium and diets were stirred well. Gentamicin solution (0.5%, v/v, from Sigma, St. Louis, MO) and antibiotic-antimycotic solution (1.0%, v/v, Sigma) were added to prevent microbial contamination.

Collection of Parasitoid Eggs

Eggs of *E. puttleri* were collected from super-parasitized CPB eggs (each beetle egg contained more than one parasitoid egg) that had been exposed to female parasitoids for approximately 1 h. In order to allow sufficient time for embryonic differentiation, parasitoid eggs were permitted to develop in their host eggs for 18–24 h. Beetle eggs were surface-sterilized by immersing them in 95% ethanol for 1 min, and 70% ethanol for 10 min, and then rinsing them 3 times with sterile distilled water. Then beetle eggs were placed in one well of a sterile glass microscope slide that contained 0.5 ml of Grace's medium. Dissection was performed with micro-dissecting probes. Beetle eggs were carefully split at the apical portion and the parasitoid eggs were liberated by gently squeezing the CPB egg to avoid damaging the wasp eggs. Parasitoid eggs were collected with a Pasteur pipet and immediately placed, individually, in one well of a 96-well flat bottom tissue culture plate (Corning Glass Works, Corning, NY) that contained artificial diet (10 µl diet per well). The outer 36 wells of the plate were filled with sterile water in order to maintain appropriate humidity. The culture plate containing *E. puttleri* eggs was maintained in a rearing chamber (prepared from a desiccator) at 24 ± 1°C, nearly 100% r.h, and a light dark regimen of L14:D10 (intensity was approximately 100 lux).

Determination of the Growth-Promoting Ability of Insect Cell Line-Conditioned Culture Medium

To evaluate the effects of cell line-conditioned medium (Table 1) on growth and development of *E. puttleri*, we prepared culture medium in which one of 12 different cell lines (Table 1) was incubated (preconditioned) for 4 days prior to its incorporation into the diet. Parasitoid eggs were removed from super-parasitized host eggs and placed in artificial diets (see previous section).

The growth-promoting ability of the cell line-conditioned media was compared and those in which *E. puttleri* developed to prepupae and/or pupae were selected for further study. To determine the effect of preconditioning time on the parasitoid, culture medium was preconditioned with each of the selected cell lines for 4, 5, or 6 days prior to use. While it would have been useful to monitor cell counts during conditioning, as stated previously, this was difficult or not feasible.

Tests were also conducted to determine how storage at cold temperature influenced the growth-promoting ability of the preconditioned medium. Cell line IPLB-CPB2 (from embryos of CPB) was used for these experiments. Medium was conditioned for 4 days prior to incorporation into the diet, and the diet was stored at 4°C for 7 or 14 days before use.

Since insect cells may have a direct effect on the developing parasitoid, the effect of cell-free vs. cell-line-conditioned media was also evaluated. Cell-free medium was prepared by centrifuging conditioned medium at 2,000*g* for 5 min and then adding the supernatant to the artificial diet.

Diets made of egg yolk and fresh cell culture medium (medium not conditioned with cell lines) were used as controls. Ex-Cell 400 (JRH BioSciences, Lenexa, KS) was used for culturing

cell lines IPLB-DU182A, IPLB-LdElta, IPLB-Tcon1, IPLB-Tex2, and IPLB-LdFB; TNM-FH (Sigma BioSciences, St. Louis, MO) was for culturing cell lines IPLB-CPB2, IPLB-PiE, and IAL-TN-R^2, and TC-100 (Sigma) was for culturing cell lines IPLB-HvE6s, IPLB-OIE505s, IPLB-PxE2, and UFL-AG-286 (Table 1).

All experimental procedures were conducted in a laminar flow hood and all equipment and materials were sterile. Cultures of *E. puttleri* were observed daily for a 30–40-day period and each experiment was replicated at least two times. The stage of development for each parasitoid was recorded, and as a measure of growth, the maximum average length of the parasitoids was determined. Only embryos that survived the first day in culture were included in the tabulations. Percent first instar was calculated by dividing the number of first instar parasitoids by the initial number of embryos that survived after one day in culture. Percent survival for second and third instar larvae, prepupae, and pupae also were calculated as the fraction of individuals that attained each of these stages. One-way analysis of variance (ANOVA) was used to analyze the data. Percent values were transformed using an arcsin square root transformation {y = arc sin [square root (x)]} to avoid bias (Hoshmand, 1994). When *F* tests were significant, the Student's *t*-test or the Fisher's Least Significant Difference Test (LSD) was utilized to analyze differences in the effects of the various insect cell lines.

RESULTS

Effects of Various Insect Cell Line-Conditioned Media

The effects of different insect cell line-conditioned media on the in vitro growth and development of *E. puttleri* are presented in Table 2. The parasitoid showed a positive response to all cell line- conditioned media tested. The media conditioned with cell lines derived from insect embryos were more effective in promoting the growth and development of the parasitoid than those conditioned with cell lines derived from fat body (IPLB-LdFB) and ovaries (UFL-AG-286) (Table 2). Thus, *E. puttleri* developed better in the diet containing the medium conditioned with cell line IPLB-LdElta (from gypsy moth embryos) than in the diet containing the medium conditioned with IPLB-LdFB, the cell line from the gypsy moth fat body (Table 2).

Medium conditioned with cell lines derived from embryos of *Lymantria dispar* (IPLB-LdElta), *Trichogramma confusum* (IPLB-Tcon1), *Trichoplusia ni* (IAL-TN-R^2), and *Plodia interpunctella* (IPLB-PiE) supported *E. puttleri* development only to the prepupal stage; but wasp pupae were

TABLE 2. Effects of Various Insect Cell Lines on the In Vitro Growth and Development of *Edovum puttleri**

Cell line	Initial no. of eggs	1st instar (%)	2nd instar (%)	3rd instar (%)	Prepupa (%)	Pupa (%)	Mean larval length (mm)
Control-1 (Ex-Cell 400)	212	91.5^{ab}	65.0^{e}	7.2^{de}	0.0	0.0	$0.65^{cde} \pm 0.24$
IPLB-DU182A	56	83.9^{b}	89.4^{bcd}	8.5^{de}	0.0	0.0	$0.60^{de} \pm 0.22$
IPLB-LdElta	50	100.0^{a}	100.0^{a}	18.0^{cd}	8.0^{c}	0.0	$0.75^{bcd} \pm 0.48$
IPLB-LdFB (F)	42	100.0^{a}	88.1^{bcd}	14.3^{de}	0.0	0.0	$0.63^{de} \pm 0.16$
IPLBTcon1	50	88.0^{b}	77.3^{de}	18.2^{cd}	9.1^{c}	0.0	$0.77^{bcd} \pm 0.36$
IPLB-Tex2	46	87.0^{b}	80.0^{cde}	0.0	0.0	0.0	$0.52^{e} \pm 0.07$
Control-2 (TNM-FH)	166	95.2^{a}	89.2^{bcd}	10.8^{de}	0.0	0.0	$0.69^{cde} \pm 0.34$
IPLB-CPB2	48	95.8^{a}	91.3^{bcd}	60.9^{a}	43.5^{a}	8.7	$1.47^{a} \pm 0.78$
IAL-TN-R^2	48	100.0^{a}	100.0^{a}	33.3^{bc}	14.8^{bc}	0.0	$0.88^{bc} \pm 0.52$
IPLB-PiE	52	100.0^{a}	96.1^{ab}	46.2^{ab}	26.9^{ab}	0.0	$0.91^{b} \pm 0.38$
Control-3 (TC-100)	222	86.5^{b}	82.3^{cd}	1.6^{f}	0.0	0.0	$0.54^{de} \pm 0.09$
UFL-AG-286 (O)	56	100.0^{a}	92.9^{abc}	7.1^{de}	0.0	0.0	$0.58^{de} \pm 0.12$
IPLB-HvE6s	54	100.0^{a}	92.6^{bc}	7.4^{de}	0.0	0.0	$0.61^{de} \pm 0.11$
IPLB-OIE505s	60	100.0^{a}	93.3^{ab}	0.0	0.0	0.0	$0.52^{e} \pm 0.08$
IPLB-PxE2	54	100.0^{a}	77.8^{de}	5.6^{ef}	0.0	0.0	$0.58^{de} \pm 0.13$

*All cell lines were incubated in a growth chamber (24 ± 1°C) for 4 days prior to diet preparation. Cell line derived from fat body = (F). Cell line derived from ovaries = (O). Controls were commercial insect cell culture media not conditioned with cell lines. Control diets consisted of 70% fresh chicken egg yolk and 30% fresh culture medium, while test diets consisted of 70% egg yolk and 30% cell line-conditioned medium (cells were removed by centrifuging at 2,000*g* for 5 min). ANOVA and LSD tests were used to test significance. Comparisons for each stage (columns) were made among diets containing medium conditioned with various cell lines. Means with different letters indicate significant differences ($P = 0.05$). For additional information, see Materials and Methods.

observed with medium conditioned with IPLB-CPB2. When cultured in the diet containing IPLB-CPB2-conditioned medium, 95.8% of the parasitoid larvae hatched, 91.3% molted to the second instar, 60.9% molted to the third instar, 43.5% became prepupae, and 8.7% pupated (Table 2). The larvae also attained a significantly greater body length (1.47 ± 0.78 mm) when fed on the diet containing IPLB-CPB2-conditioned medium (Table 2). Interestingly, the medium conditioned with the cell line, IPLB-Du182A, derived from embryos of *Diabrotica undecimpunctata,* an insect that like CPB belongs to the family Chrysomelidae, did not appear to be beneficial to the parasitoid (Tables 1, 2). It is possible that differences in the medium used for culturing the cell lines (Ex-cell 400 for IPLB-DV182A, and TNM-FH for IPLB-CPB2) could be responsible for the inability of IPLB-DV182A-conditioned medium to support parasitoid development.

Effects of Preconditioning Time

Depending upon the cell line used and the wasp instar examined, the duration of preconditioning was a critical factor in promoting wasp growth and development (Table 3). Five days of preconditioning tended to result in greater growth and development of *E. puttleri* than 6 days (and often than 4 days) of preconditioning. This effect was most apparent (statistically significant) when cell line, IPLB-LdElta, (derived from gypsy moth embryos) was tested. When preconditioning was conducted for 4 days, only 16% of the parasitoids developed to the 3rd instar, 4% reached the prepupal stage, and no pupae were observed. After preconditioning for 5 days, however, 41.7% of the parasitoids molted to the 3rd instar, 25% developed to the prepupa, and 4.2% reached the pupal stage. Also, with the longer preconditioning time, parasitoid size increased from 0.75 ± 0.48 mm (4 days) to 1.03 ± 0.54 mm (5 days) (Table 3). This beneficial effect decreased when preconditioning time was prolonged to 6 days (Table 3). For IPLB-CPB2 (from CPB embryos), percentages of 3rd instars and prepupae were higher (although not significantly higher) when medium was conditioned for 5 days as compared to 4 or 6 days.

Effects of Artificial Diets With or Without Insect Cells

In general, the presence or absence of cells in the conditioned medium that was added to the artificial diet did not appear to have a great impact in promoting the growth and development of *E. puttleri* (Table 4). Some cell line-conditioned media performed better when cells were present,

TABLE 3. Effects of Preconditioning of Cell Lines on the In Vitro Growth and Development of *Edovum puttleri**

Cell line	Initial no. of eggs	1st instar (%)	2nd instar (%)	3rd instar (%)	Prepupa (%)	Pupa (%)	Mean larval length (mm)
IPLB-CPB2							
4 days	48	95.8[a]	91.3[b]	60.9[a]	43.5[a]	8.7[a]	1.47[a] ± 0.78
5 days	58	100.0[a]	100.0[a]	75.9[a]	60.3[a]	10.4[a]	153[a] ± 0.81
6 days	54	100.0[a]	96.3[ab]	64.8[a]	48.2[a]	9.3[a]	1.48[a] ± 0.69
IAL-TN-R[2]							
4 days	48	95.8[a]	95.7[ab]	32.6[a]	15.2[b]	0.0	0.88[ab] ± 0.52
5 days	52	100.0[a]	100.0[a]	48.1[a]	34.6[a]	0.0	1.01[a] ± 0.27
6 days	54	100.0[a]	88.9[b]	7.4[b]	3.7[c]	0.0	0.66[b] ± 0.50
IPLB-PiE							
4 days	52	100.0[a]	96.1[ab]	46.2[a]	19.2[a]	0.0	0.91[ab] ± 0.38
5 days	44	100.0[a]	100.0[a]	50.0[a]	27.2[a]	0.0	1.04[a] ± 0.51
6 days	34	100.0[a]	91.2[b]	20.6[b]	14.7[a]	0.0	0.75[b] ± 0.43
IPLB-LdElta							
4 days	50	100.0[a]	96.0[ab]	16.0[b]	4.0[b]	0.0	0.75[b] ± 0.48
5 days	48	100.0[a]	100.0[a]	41.7[a]	25.0[a]	4.2	1.03[a] ± 0.54
6 days	48	100.0[a]	87.5[c]	8.3[b]	0.0	0.0	0.71[b] ± 0.29
IPLB-Tcon1							
4 days	50	88.0[b]	100.0[a]	18.2[a]	9.1[a]	0.0	0.77[a] ± 0.36
5 days	56	100.0[a]	96.4[a]	28.6[a]	10.7[a]	0.0	0.73[a] ± 0.33
6 days	46	95.7[ab]	95.5[a]	0.0	0.0	0.0	0.57[a] ± 0.09

*Diets consisted of 70% fresh chicken egg yolk and 30% cell line-conditioned (4, 5, or 6 days) medium. Differences in the effects among the three cell-line-preconditioning times were compared. ANOVA and LSD tests were used to test significance. Means with different letters indicate significant differences ($P = 0.05$).

TABLE 4. Development and Growth of *Edovum puttleri* in Artificial Diets That Either Contain Cells or Are Cell Free*

Cell line	Initial no. of eggs	1st instar (%)	2nd instar (%)	3rd instar (%)	Prepupa (%)	Pupa (%)	Mean larval length (mm)
IPLB-CPB2							
With cells	58	100.0[a]	100.0[a]	75.9[a]	60.3[a]	10.4[a]	1.53[a] ± 0.81
Without cells	56	100.0[a]	98.2[a]	69.6[a]	44.6[a]	7.1[a]	1.49[a] ± 0.86
IAL-TN-R[2]							
With cells	52	100.0[a]	100.0[a]	48.1[a]	34.6[a]	0.0	1.01[a] ± 0.27
Without cells	52	100.0[a]	92.2[a]	38.5[a]	15.4[b]	0.0	0.51[b] ± 0.09
IPLB-PiE							
With cells	44	100.0[a]	100.0[a]	50.0[a]	27.3[a]	0.0	1.04[a] ± 0.51
Without cells	44	100.0[a]	100.0[a]	50.0[a]	36.4[a]	0.0	0.98[a] ± 0.35
IPLB-LdElta							
With cells	48	100.0[a]	100.0[a]	41.7[a]	25.0[a]	4.2[a]	1.03[a] ± 0.54
Without cells	56	100.0[a]	100.0[a]	75.0[b]	39.3[a]	7.1[a]	1.28[b] ± 0.40
IPLB-Tcon1							
With cells	56	100.0[a]	96.4[a]	28.6[a]	10.7[a]	0.0	0.73[a] ± 0.33
Without cells	48	95.8[a]	82.6[a]	30.4[a]	4.4[a]	0.0	0.73[a] ± 0.28

*All media were preconditioned with cell lines for 5 days. Cells were removed by centrifuging at 2,000*g* for 5 min. Diets consisted of 70% fresh chicken egg yolk and 30% cell-line-preconditioned media. Differences in the effects between the diets (either contained cells or were cell-free) were compared. ANOVA and Student's *t*-test were used to test significance. Means with different letters indicate significant differences ($P = 0.05$).

while for others, the results were reversed (Table 4). Thus, although the difference was not significant, when cells of the IPLB-CPB2 were present in the conditioned medium, the parasitoid exhibited better development (60.3% reached prepupal stage and 10.4% pupated) than when cells were absent (44.6% reached the prepupal stage and 7.1% pupated). However, for the cell line IPLB-LdElta, the diet prepared with cell free-conditioned medium was more effective (39.3% reached prepupal stage and 7.1% pupated) in promoting the development of the wasp than the diet prepared with conditioned medium that contained cells (25.0% reached the prepupal stage and 4.2% pupated) (Table 4).

Effects of Cold Storage on the Stability of Putative Growth Factors

For the insect cell lines tested, growth factors released into the culture medium were not stable when stored at 0–4°C for 7 or 14 days; their growth-promoting effect was reduced (Table 5). The percentage of larvae molting sharply declined and no pupae were observed (Table 5). Furthermore, the longer the time of exposure to cold temperature, the greater the decrease in percent prepupal and pupal formation. The presence or absence of cells also influenced the results. Thus, in the diet prepared with cell free-conditioned medium, even after storage for 14 days at 0–4°C, the medium maintained its ability to support development of wasp larvae to the prepupal stage, but in the diet prepared with conditioned medium that contained cells, after exposure to cold temperature, the wasp larvae never reached the prepupal stage (Table 5). Storage of control media (growth factors absent) at cold temperatures, however, did not have a significant adverse effect on wasp development.

DISCUSSION

The importance of growth factors from host insects in promoting the development and growth of parasitoids in vitro has been recognized for years (Nettles, 1990). However, the growth factors upon which most entomologists focused were derived from host hemolymph or tissues (Vaughn and Louloudes, 1978; Ferkovich et al., 1987, 1990; Irie et al., 1987; Ferkovich and Dillard, 1990; Greany et al., 1990; Nettles, 1990; Wielgus et al., 1990). Although continuous cell lines from more than 100 insect species have been established during the last three decades (Lynn, 1996), little attention was paid to the benefits of using growth factors derived from insect cell lines for the in vitro rearing of parasitic insects (Ferkovich and Oberlander, 1991). As a result, growth factors have not been purified or characterized, and their mode of action in promoting development has not been studied.

TABLE 5. Effects of Cold Temperature (0–4°C) on the Stability of Growth Factors in the CPB Embryonic Cell Line*

Cell line	Initial no. of eggs	1st instar (%)	2nd instar (%)	3rd instar (%)	Prepupa (%)	Pupa (%)	Mean larval length (mm)
Control (TNM-FH)							
Fresh	50	96.0[a]	93.3[a]	25.0[a]	16.7[a]	0.0	0.74[a] ± 0.46
7-day	56	96.4[a]	96.3[a]	29.6[a]	11.1[a]	0.0	0.83[a] ± 0.46
14-day	58	94.8[a]	89.1[a]	21.8[a]	12.7[a]	0.0	0.79[a] ± 0.51
With cells							
Fresh	48	95.8[a]	91.3[a]	60.9[a]	43.5	8.7	1.47[a] ± 0.78
7-day	52	92.3[a]	91.7[a]	4.2[b]	0.0	0.0	0.63[b] ± 0.20
14-day	22	90.9[a]	80.0[a]	0.0	0.0	0.0	0.61[b] ± 0.10
Without cells							
Fresh	50	96.0[a]	87.5[a]	48.0[a]	39.6[a]	4.2	1.33[a] ± 0.75
7-day	48	93.8[a]	95.6[a]	31.1[ab]	26.7[ab]	0.0	1.02[b] ± 0.59
14-day	54	92.6[a]	96.0[a]	16.0[a]	12.0[b]	0.0	0.78[c] ± 0.50

*CPB embryonic cells were incubated in medium (in a growth chamber at 24 ± 1°C) for 4 days prior to diet preparation. Cells were removed by centrifuging at 2,000*g* for 5 min. Control diet consisted of 70% fresh chicken egg yolk and 30% TNM-FH medium, while test diets consisted of 70% fresh chicken egg yolk and 30% cell-line-conditioned medium. Differences in the effects among the three treatments were compared. 7-day = 7 days at 0–4°C; 14-day = 14 days at 0–4°C. ANOVA and LSD tests were used to test significance. Means with different letters are significantly different ($P = 0.05$).

Ferkovich et al. (1991, 1994) were the first to report the positive effect of medium conditioned with a cell line from *Lymantria dispar* in stimulating the embryonic development of *M. croceipes* in vitro. In the studies reported here, the effects of medium conditioned with several cell lines derived from various insect species (including Coleoptera, Hymenoptera, and Lepidoptera) and tissues (including embryos, fat body, and ovaries) in promoting the in vitro growth and development of the egg parasitoid *E. puttleri* were studied. The observation that culture media conditioned with these insect cell lines were capable of promoting wasp growth and development suggests that they produce important growth factor(s). However, cell conditioning may also exert a positive affect by altering amino acid content, shifting pH or releasing degradative enzymes into the medium. The ability of mammalian cells to produce growth factors that enhance the proliferation of other unrelated cells is well established (Gospodarowicz and Moran, 1976). For example, a growth factor produced by L-cells induces macrophages to multiply. Therefore, it seemed reasonable to assume that growth factors produced by insect cell lines could stimulate the growth and development of parasitoids that are cultured in vitro.

When cell lines from various origins or sources were used to condition culture medium, significant differences were observed in the development of *E. puttleri* based on the cell lines selected (Table 2). Our results, then, are similar to those reported by Ferkovich et al. (1994). Embryos of *M. croceipes* responded positively to the medium conditioned with cell lines derived from *Trichoplusia ni* and *L. dispar* (both insects are atypical hosts of the parasitoid), but not to the medium conditioned with cell lines from *Spodoptera frugiperda* or *Heliothis virescens*. (the natural hosts of the parasitoid). As expected, since the wasp is an egg parasitoid, cell lines derived from embryos were more effective in promoting the growth and development of *E. puttleri* than medium conditioned with cell lines derived from fat body and ovaries. Growth factors produced by embryonic cell lines should be more suitable for the parasitoid than growth factors produced by other types of cell lines. This difference may be caused, in part, by receptor specificity for particular growth factors. In *Platycnemis* (Odonata), the anterior differentiation center of the embryos was activated by a growth factor released by the posterior differentiation center (Chapman, 1982). The differentiation of *H. virescens* midgut cells was stimulated by two growth factors released by a cell line from *M. sexta* midgut (Loeb, personal communication). Receptors in the developing parasitoid may be more sensitive/responsive to the growth factors from embryonic cell lines than from those prepared from fat body or ovaries.

When compared to media conditioned with other embryonic cell lines, the medium conditioned with IPLB-CPB2, the cell line derived from embryos of the Colorado potato beetle (the natural host of *E. puttleri*) was superior in promoting the development of *E. puttleri* from the egg to the

pupal stage (Table 2). These results suggest that growth factors produced by this cell line could be specific for the wasp and are similar to the growth factors supplied by the natural host. Furthermore, the observation that *E. puttleri* grew and developed better when the conditioned medium added to the diet contained cells from some cell lines than when cells were absent (Table 4), indicates that the cells themselves may have a role in providing nutrients or in creating other environmental conditions (e.g., pH and osmotic pressure) that are conducive to wasp development. However, since for the majority of cell lines tested, the presence of cells in the conditioned medium did not have a significant effect on the promotion of *E. puttleri* development, it must be the growth factors released, rather than the cells themselves, that are of prime importance for the wasps to develop through the pupal stage.

Effects of growth factors appear to be species-specific. Thus, although *D. undecimpunctata* belongs to the same family (Chrysomelidae) as the CPB, medium conditioned with the IPLB-DU182A (from embryos of *D. undecimpunctata*) cell line is not effective in promoting the growth and development of *E. puttleri* (Table 2). Alternatively, since all the embryonic cell lines were developed from whole embryos, the actual cell type in culture could (and probably does) vary between the lines tested. Thus, our results could be due to the actual cell type in the various lines.

Our present study suggests that the duration of preconditioning with insect cell lines may be critical in producing conditioned medium, which, when added to the diet, will promote the best growth and development of the parasitoid. Five days of preconditioning tended to be optimum (Table 3). Shorter or longer times often reduced the beneficial effects of cell line conditioning. Shorter times may not have allowed the cell lines to release a sufficient amount of growth factor(s), while longer times may have permitted the cell lines to produce too much growth factor(s), which could be detrimental to wasp development. Diluting may help solve this problem.

In general, growth factors have been found to be peptidic in nature (Hardie, 1991). Thus, it is not surprising that, in our experiment, growth-promoting activity was lost after artificial diets were subjected to extended periods of storage, even under conditions of refrigeration. Long exposure time may result in structural modifications, which, in turn, cause biological activity to be lost or reduced. Since the diet containing conditioned medium devoid of insect cells was more tolerant to storage at cold temperature, it is possible that during extended periods of storage, cells released toxic substances into the diet and/or that cells died and the dead cell bodies were toxic to the developing parasitoid.

Growth factors are typically peptides that promote or inhibit the growth of cells depending on the state of development or differentiation of the target cells (Sporn and Roberts, 1991). In vertebrates, the mode of action of growth factors has been well studied (Gospodarowicz and Moran, 1976). However, little is known about the mechanism of action of insect growth factors, especially for in vitro-reared parasitoids. Therefore, in future studies, it is important to explore two areas in order to understand how growth factors released into the culture medium by insect cell lines function. First, insect growth factor(s) must be isolated and characterized, and, second, receptors must be identified and the process of activation must be elucidated.

In summary, the most useful cell lines for generating conditioned media that promote the in vitro growth and development of *E. puttleri* have been identified. Medium conditioned with the IPLB-CPB2 cell line, derived from CPB embryos, is as effective as *M. sexta* larval hemolymph in stimulating development of the wasp through the pupal stage (Hu et al., 1998). Parameters for preconditioning time and storage have been determined. Five days of preconditioning appear to be optimal for most cell lines and storage at cold temperature is best when diet is prepared with cell-free conditioned medium. Our studies demonstrate that insect cell line-conditioned medium can replace insect hemolymph and can be incorporated into artificial diet for the mass rearing of *Edovum puttleri* in vitro. Since techniques for the large-scale production of insect cell lines are available, the cost of producing/using cell line-conditioned medium should be lower than that of using insect hemolymph. Purification and characterization of growth factors released by the insect cell lines into the medium should facilitate the development of a successful in vitro rearing system for *E. puttleri* and/or other insect parasitoids.

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